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(54) Abstract Title
Method of biological control using a lethal genetic system

(57) A method is disclosed for the control of insects using a dominant sex-specific lethal genetic system which is conditional i.e. expressed when the insect is in its natural environment. The system may be conditional on temperature or a dietary additive which suppresses expression when supplied to insects in the laboratory. This suppression is removed once the insect is in its natural environment where the additive is not found in its food.

The lethal effect may be expressed in the laboratory or natural environment so that only one sex e.g. males is released or survives to interbreed with the wild population thus passing on the genetic system. Alternatively, the lethal system may be sex-specific in an adult organism but be lethal to both males and females in the larval stage.

The method can be applied for the control of plants wherein one sexual entity of a plant is killed.

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Biological Control

The present invention relates to a method for controlling the population of an organism.

BACKGROUND OF THE INVENTION

Methods of biological control are known for insects and plants. One method currently employed for the control of insect populations is termed the "sterile insect technique" (SIT), also known as the "sterile insect release method" (SIRM). In this method, sterile males are released into the environment, wherein they compete with the wild-type (fertile) males for mates. Females which mate with sterile males produce no offspring, and the release of large numbers of sterile males, therefore, leads to a decrease in the size of the next generation. In this way the size of the wild population is controlled.

SIT requires some mechanism for insect sterilisation. In addition, SIT commonly also employs separation of males from females, with the release of only one sex. This is desirable in the case of an agricultural pest, such as the medfly, where the female damages fruit, even if the female is sterile. Similarly, only the female mosquito bites humans. As such, release of the female fly is preferably avoided in these cases.

Current techniques to achieve both sterilisation and separation of the sexes all have drawbacks. In some cases it is possible to separate males and females by criteria such as pupal mass, time of eclosion etc, but these methods are unlikely reliably to yield a truly single-sex population. Separation of males and females also often involves the use of mutagenesis, to induce a visible or otherwise selectable difference between the sexes, but mutagenesis can reduce the fitness of the resultant stock with respect to the wild type, which is undesirable.

Fitness may be further reduced in the sterilisation procedure, in which insects are given a sterilising dose of radiation (X rays or gamma rays), or are chemically sterilised. Frequently, the doses of chemicals or the dose of radiation required to induce sterilisation are very similar to that which is lethal for the organism. As such, sterile organisms are frequently impaired in their

ability to mate. Furthermore, both chemical and irradiation methods utilise technologies which are not specific to the target organism, with consequent potential danger to workers. Both methods produce an environmental hazard, as the irradiation source or chemicals will need to be disposed of. In addition, there are inherent dangers and additional labour costs in the use of an irradiation source such as a strontium source.

Fryxell and Miller (Journal of Economic Entomology, Vol 88, No 5, pages 1221 - 1232) disclose an alternative strategy for insect control, using *Drosophila* containing a dominant conditional lethal gene which is expressed under appropriate cold conditions in the wild.

DeVault *et al.* (Biotechnology, Vol 14, January 1996, page 46-49) disclose a two-stage process which is a modification of the SIT procedure. Insects are initially separated by expression of a stably inserted female specific promoter linked to a lethal gene, which is expressed to kill females and to produce just one sex. The remaining males can then be sterilised by irradiation or chemical treatment and released into the environment. However, this method suffers from the drawback referred to above, in that released flies have reduced fitness due to the sterilisation treatment. Alternatively, the DeVault article discloses use of this genetic sexing step in combination with a second genetic system, which may serve to sterilise or retard the hardiness of the natural population.

SUMMARY OF THE INVENTION

We have now discovered a new method for biological control, applicable to organisms capable of sexual reproduction, wherein only one lethal genetic system is required, the expression of which is used in both sex separation and biological control. The lethal genetic system is a conditional dominant sex-specific lethal genetic system, which is expressed in the restrictive conditions of the natural environment of an organism. However, the expression of the lethal genetic system may be controlled under permissive conditions in a laboratory, factory or other regulated system, for example, to allow growth of a normal populations, e.g. insect stock with both sexes. Prior to release of the factory or laboratory stock into the environment the conditions can be manipulated to ensure only single sex populations of the organism are

distributed into the environment. No additional irradiation of the organism is required and the arrangement removes any requirement for use of two separate genetic systems (i.e. those employed by De Vault *et al.* for sexing and, for example, sterilisation). Only one genetic system needs to be constructed and inserted into the organism, which renders the methodology easier and quicker.

Thus, in accordance with the present invention, a multicellular organism carries a dominant sex-specific lethal genetic system which is conditional, and does not have a dominant sex-specific lethal genetic system which is unconditional and is expressed in every individual.

Specifically, under permissive conditions, the lethal genetic system in the organisms of this invention is not expressed, and a stock of organisms can be bred. Imposition of restrictive conditions then allows one sex (for example, females) to be killed. The remaining sex (males) can be released to the environment, and the genetic system is passed on to at least some offspring resulting from any sexual reproduction between said males and a wild-type organism of the same species. The conditional dominant lethal genetic system is selected such that expression of the lethal system occurs in the natural environment. As a result, for a female specific lethal genetic system, all females which result from the mating are then killed, while the males survive to pass on the system to the next generation in a proportion of cases. In this way, biological control is achieved.

If desired, the stock of organisms grown under permissive conditions can be released into the environment, without imposing the restrictive conditions to kill off one sex before release. This variation permits the possibilities of using a timing mechanism, e.g. life cycle stage, in creating a biological control agent. That is, the imposition of the restrictive condition is programmed by an event other than, for example, a pre-determined change in factory / laboratory conditions prior to release into the environment. For example, release of a normal population of larvae creates a useful time-scatter or delayed release agent. By this is meant that individual larvae may proceed to maturity at different rates and therefore release of the single sex genetically engineered population could occur over a period of time and hence create a maximum probability of interaction with sexually active wild populations over that period. This aspect may have advantages over a single time point release of a single sex population of the

genetically engineered adults. There are other advantages, notably that the last (biggest) generation does not have to be reared in the factory, laboratory or other regulated environment, so saving space and food and thereby giving a more economic process. Moreover, the released larvae will compete with the larvae of the wild population, increasing mortality through density-dependent mechanisms. By way of illustration, this variation might be useful with mosquitoes, where the larvae are harmless, but not with medfly, where the larvae eat fruit.

Therefore, in a first aspect the present invention provides a method of biological control for an organism, the organism having discrete sexual entities, the method comprising the steps of:

1 production of a stock of genetically engineered organism;

2 release of the genetically engineered organism into the environment either as

a) a normal population (i.e. containing both sexes) at a certain stage of the life cycle of the organism, e.g. larvae, in the knowledge that females will die and only males will mature into adults, or

b) a single sex population, i.e. after the sex specific dominant lethal effect has been expressed prior to release.

The invention relies on expression of a conditional dominant lethal genetic system capable of sex specific lethality, in order to eliminate one sexual entity. The conditional expression of the lethal gene is such that the lethal effect occurs in the natural environment of the organism to cause the biological control.

In a second aspect of the invention, the invention accordingly involves a third step;

3 allowing biological control to occur.

The invention further provides a method of biological control, comprising:

breeding a stock of males and female organisms under permissive conditions, allowing the survival of males and females, to give a dual sex biological control agent;

optionally before the next step imposing or permitting restrictive conditions to cause death of individuals of one sex and thereby providing a single sex biological control agent comprising individuals of the other sex carrying the conditional dominant lethal genetic system;

releasing the dual sex or single sex biological control agent into the environment at a locus for biological control, and

achieving biological control through expression of the genetic system in offspring resulting from interbreeding of the individuals of the biological control agent with individuals of the opposite sex of the wild population.

The invention further provides a method of biological control in which the growth of a stock of organisms under permissive conditions, once initiated, is self-sustaining and requires no additional pool of organisms for its maintenance.

The invention further provides a method of biological control in which the expression of the lethal genetic system occurs in the absence of a substance which is absent from the natural environment of the organism, thus ensuring effective biological control when the organism is released.

General Description of the Invention

When a single sex biological control agent is desired, separation of the sexual entities is normally achieved in the method by removal of permissive conditions while a stock of an organism is grown up, resulting in the sex specific lethal effect of the genetic system being manifested. A single sex population remaining may then be isolated.

We prefer that the lethal effect is female specific. However, a male specific lethal effect may be required in certain situations. With reference to plants, the sexual entities need not be discrete organisms, but parts of the same organism. The present invention may thus also be applied to plants, wherein one sexual entity of a plant is killed. With a single sex biological control agent, the conditional dominant lethal genetic system is permitted to be expressed during growth cycles before release, and the plant then distributed. Alternatively, no such permissive

expression might be needed before release, for instance in the case of seed distribution with the lethal effects only manifesting once the plant reaches a certain further stage in its life cycle in the environment.

With respect to insects and other animals, distributing the organism typically occurs by release of the organism into the environment. With plants, distributing typically occurs by planting of mature plants, seedlings or seeds, or any suitable form of the organism in the environment.

The lethal genetic system is suitably comprised of a lethal gene and controlling and/or regulatory elements. However, in one embodiment, the lethal system may be comprised simply of a lethal gene, sufficient to produce the lethal effect.

The dominant genetic system suitably includes a dominant gene whose effect is phenotypically expressed in the heterozygous state. This dominant effect ensures that, if an organism only receives one copy of the lethal genetic system, then the lethal effect of that system will nevertheless be exerted in the host in the natural environment of the organism.

The conditional effect of the dominant lethal genetic system is seen except under defined permissive conditions. In the present invention the restrictive conditions occur in the natural environment of the organism, and are those conditions which allow the lethal effect of the lethal system to be expressed. The permissive conditions which allow the survival of the organism are only present when adopting permissive conditions in the regulated growing environment.

The natural environment of the organism is generally the environment in which the population to be controlled is located, or may survive. Additionally, the natural environment is also an environment which provides the necessary restrictive conditions.

DETAILED DESCRIPTION OF THE INVENTION

The lethal genetic system of the present invention may be any genetic element or combination of elements which is capable of producing a lethal effect. We prefer that the lethal

genetic system comprises a DNA sequence encoding a potentially lethal gene product (a lethal gene) and controlling elements such as promoters, enhancers or trans-activator components. The elements which regulate the gene may be located on the same chromosome as the lethal gene, which is preferred, or on a different chromosome. We particularly prefer that the lethal system is a lethal gene the expression of which is under the control of a repressible transactivator protein. In an alternative embodiment the lethal system may simply be the lethal gene alone, or in combination with its native promoter.

The lethal effect of the lethal system may affect the whole organism, or be targeted to specific tissues within an organism. For example, in plants the lethal effect may be targeted to only a part of the host plant, such as one of the sexual organs of the plant. As such, in the present invention, a reduction in the wild type population size is achieved without the use of applied sterilisation by externally applied agents such as irradiation or chemicals, but through the use of targeted lethality based on zygotic lethality.

The lethal gene may be any genetic element which is capable of causing the death of, or leading to the fatality of, the host. In particular, the term covers gene fragments capable of exerting a lethal effect, and is not limited to full length genes. Any element capable of exerting a lethal effect which may be conditionally controlled is covered by this term.

The choice of dominant lethal gene is not critical to the invention. There is a wide range of suitable gene products, with varying toxicities. For example, dominant mutant forms of cell-signalling or cell-cycle genes are appropriate for use in the present invention. Constructs which result in overexpression of such genes may also be lethal. Similarly constructs which result in inadequate expression of any essential gene would also be lethal. This might be achieved by expression of an inhibitory sequence, for example antisense RNA, sense RNA (acting by gene silencing), double stranded RNA ("inhibitory RNA" or RNAI) or other inhibitory RNA molecule. Overexpression of protein inhibitors of essential functions could also perform this lethal function. Other suitable targets for engineering constructs include genes which disrupt metabolism or regulation of the cell to a fatal extent, such as disruption or overexpression of extracellular signalling factors such as functional homologues of Wnt, Shh or TGFβ. Where highly toxic gene products are used, such as diphtheria toxin and ricin A, we prefer that the

genes are only expressed at levels sufficient to kill the organism, but with minimum environmental impact.

Conditional

The conditional nature of the lethal system allows recombinant organisms to be bred under conditions permissive for organism survival, for example in a factory or laboratory, and then released into the natural environment. The lethal effect of the lethal system is controlled such that the released organisms are able to breed, and sexual reproduction allows the lethal system to be passed into the wild type population, killing all or a defined group of these organisms. We prefer that the lethal effect results in killing of greater than 90% of the target class of the progeny of matings between released organisms and the wild population. The target class is for example females, i.e. 50% of the progeny. More preferably the lethal effect results in killing of greater than 95% of the target class, and most preferably 100 % of the target organisms in the environment.

The conditional nature of the lethal system may be conditional on any suitable factor, such as temperature, diurnal cycle (with light duration and/or intensity being factors) or pheromones, for example. In this case, the recombinant stock could be reared at the permissive temperature, and released into an environment having a restrictive temperature. However, we prefer that the lethal effect of the lethal system is conditional upon a dietary additive, such as a food or water additive, which is not a normal food component for the target species. This allows the recombinant stock to be grown on food or water containing the additive, which prevents the lethal effect. On release into the wild, the organism has no exposure to the additive, and the lethal effect of the lethal system is expressed in the progeny of a mating with the recombinant organism of the invention. It may also be expressed in the parent organism under certain circumstances, although the released organism must survive long enough to mate.

Where the lethal effect is conditional upon a dietary additive, it may be that the progeny will survive without the dietary additive. For example, the progeny might retain sufficient of the additive without feeding, or at the least the additive may be slowly lost from the progeny. This

effect might pass through one or more generations before the lethal effect is fully expressed under restrictive conditions.

We prefer that the recombinant multicellular organism of the present invention contains a dominant lethal system the lethal effect of which is conditionally suppressible. In this way, the lethal effect is suppressed under controlled conditions, but not suppressed in the natural environment of the organism. However, there may be other ways to attain conditional expression (for example, conditional activation), any of which may be used in the present invention.

It is the lethal effect of the lethal system which is conditional, and not solely the expression of the lethal gene. Therefore, the invention includes the possibility of conditional control both at the level of lethal gene expression, and by control of the activity of the lethal gene product. As such, the invention includes the case in which the lethal gene product is being produced but the effect of which is masked in some way.

We prefer that the method of the invention uses only organisms with a single conditional dominant lethal genetic system. In addition, we prefer that this system is the only recombinant element present in the organism. We particularly prefer that the organism contains only one type of lethal gene, but it is possible to envisage multiple lethal genes under the same regulatory control, giving the integrated genetic construct concept but a more efficient lethality of the system. This single lethal gene may be under the control of just one promoter in the genetic system, or more than one promoter.

Organism

A recombinant organism refers generally to any organism whose genetic material has been altered by genetic manipulation. We prefer that the organism is modified by insertion of a gene, gene fragment or genetic element (such as a promoter or enhancer) from another species, to produce a transgenic organism. The transgenic component is generally the lethal system which produces a conditional lethal effect. However, a conditional lethal effect may also be generated using genetic components derived from the same (host) species. For example, a

promoter derived from a different gene in the same species, when placed in front of a gene which is only normally expressed at low levels, may result in a lethal effect. The recombinant organism is thus either a transgenic organism or one in which the host genetic material has been modified to produce a lethal system.

The multicellular organism may be any organism, such as a plant or animal. Indeed, the invention is only limited to those organisms having a sexual component in their life cycle, which enables the lethal system to be transferred from one organism to another. We particularly prefer that the multicellular organism of the invention is an insect, with insect pests being particularly preferred. An insect pest may be either a direct or an indirect pest. Direct pests are those insects which cause damage at any stage of the life cycle by, for example, eating crops or damaging animals. The New World screw-worm fly *Cochliomyia hominivorax*, for example, is a direct pest of cattle. Indirect pests are those insects which are vectors of human diseases, such as mosquitoes which carry malaria. Indirect pests of organisms other than humans, such as livestock or plants are also known.

Preferred insect targets for the present invention include Crop (arable and forestry) pests Animal pests and Disease vectors. Examples of specific organisms which may be used in the present invention include: Japanese beetle (*Popilla japonica*),White-fringed beetle (*Graphognatus spp.*), Citrus blackfly (*Aleurocanthus woglumi*), Oriental fruit fly (*Dacus dorsalis*), Olive fruit fly *(Dacus oleae*), tropical fruit fly (*Dacus cucurbitae, Dacus zonatus*), Mediterranean fruit fly (*Ceratitis capitata*), Natal fruit fly (*Ceratitis rosa*), Cherry fruit fly *(Rhagoletis cerasi*), Queensland fruit fly (*Bactrocera tryoni*), Caribbean fruit fly (*Anastrepha suspensa*), imported fire ants (*Solenopis richteri, Solenopis invicta*), Gypsy moth (*Lymantria dispar*), Codling moth (*Cydia pomonella*), Brown tail moth (*Euproctis chrysorrhoea*), yellow fever mosquito (*Aedes aegypti*), malaria mosquitoes (*Anopheles gambiae, Anopheles stephansi*), New world screwworm (*Cochliomyia hominivorax*), Old World Screwworm (*Chrysomya bezziana*), Tsetse fly (*Glossina spp*), Boll weevil (*Anthonomous grandis*), Damsel fly (*Enallagma hageni*), Dragonfly (*Libellula luctuosa*) and rice stem borer (*Tryporyza incertulas*). General classes of plant suitable for use in the present invention include plants which are crop pests, animal pests or disease vectors. Reviews discussing the suitability of many of the above are: C. Boake *et al.*, (1996) Annu. Rev. Entomol. 41: 211-219, J. Meyers *et al.*, (1998) Annu.

Rev. Entomol. 43: 471-491, C. Calkins *et al.*, (1994) Fruit flies and the sterile insect technique. CRC Press. ISBN 0849348544, E. Krafsur *et al.*, (1997) Annu. Rev. Entomol. 42: 503-523 and R. de Shazo *et al.*, (1994) J. Allergy Clin. Immunol. 93(5): 847-850. It will be understood that the present invention is generally applicable to all multicellular organisms capable of sexual reproduction, such as plants and animals.

For all animals, the transgenic stock is released into the environment at appropriate sites and times. For plants, where the adults are not mobile, the procedure is slightly different. Either the gametes themselves are released, e.g. as pollen, or plants are dispersed, e.g. at field margins, to pollinate wild weeds and so reduce their reproductive potential. The present invention is of particular use in the control of those weeds, such as rye grass, which are not well controlled by current herbicides, or against weed types which have developed herbicide tolerance.

Not all of the terms which are used to describe, for example, plants are applicable to animals or *vice versa*. However, the principles of the invention as laid out in relation to one species may readily be applied to other species by the person skilled in the art.

Targeted lethal effect

The multicellular organism of the present invention preferably has a lethal system homozygous at one or more loci. In the situation where there is one homozygous copy of the lethal system, then at least one copy of the system will be passed to any offspring during sexual reproduction. Therefore, the dominant lethal effect will be exerted, except in permissive conditions. The present invention may be carried out using a heterozygote for the dominant lethal system. However, in this case, not all the offspring will have a copy of the lethal system, and the effect on the population is reduced.

It is preferred that all the elements of the genetic system are present on the same chromosome, in close proximity. In this way, it is likely that all elements of the lethal system are passed on to subsequent generations. However, the lethal system can also function when controlling elements are present at different genetic loci to the lethal gene, if controlling effects of these elements are exerted in *trans*, for example. In that event, the genetic system is still

effective if the controlling and lethal elements are also homozygous, and at least one copy of each is transferred to the offspring.

Sex Specific

The method of the invention uses a sex specific lethal system to achieve sex separation before or after release of organisms into the environment. In a preferred embodiment, the multicellular organism is an insect containing a homozygous dominant lethal system, the lethal effect of which is lethal only to females. In this embodiment males released into the natural environment will not be killed. After mating with females, female offspring will contain at least one copy of the dominant system and be killed. However, male offspring, 50% of which contain the dominant system, are viable and may mate with further females. In this way, the dominant system may be transmitted to subsequent generations, although without further artificial introductions the system will eventually be lost from the gene pool.

In the case in which a male contains a lethal genetic system with a female specific lethal effect, then males released into the environment will not be killed. However, the lethal effect of the lethal system is still manifested in the natural environment - even if this effect is limited to females.

Sex-specific lethality may be achieved in a number of different ways. For example, it is possible to use a sex-specific lethal gene as part of the lethal system, whose gene product is toxic only in one sex. This approach will allow killing of a single sex even if expression of the lethal gene of gene product is not sex specific. Candidates for female sex-specific lethal genes includes genes from the sex determination pathway, for example normally active only in males and toxic in females, or genes derived from sexual differentiation or gametogenesis systems.

Alternatively, expression of the lethal gene or gene product may be controlled so that it is expressed or produced only in one sex (or in only one gamete or sexual organ of a hermaphrodite). For example, sex-specific promoters or enhancers may be used, either in combination with sex-specific lethal genes or non-specific lethal genes. Sex-specific splicing provides another mode for sex-specific gene expression. All possible combinations of non-

specific lethal genes, sex-specific lethal genes, non-specific promoters and sex-specific promoters are envisaged by the present invention. In addition, other sex-specific factors which control the lethal effect of the lethal gene are included in the present invention.

Not sex specific

The present invention also includes a method of biological control in which the lethal effect may be sex-specific at one stage of the life cycle, but be lethal to both sexes at another stage. For example, the lethal system may be female specific in an adult organism, but be lethal to both males and females in the larval stage. In such a case, one sex may be killed by expression of the lethal system in the adult form. When the organism then breeds in the wild, passing on the genetic construct, then both males and females can be killed. Such an effect can be achieved by a promoter which is sex specific at one life cycle stage, but not at another, or by placing the lethal gene under control of two different promoters, for example.

For example, a lethal effect manifested at an embryonic or larval stage will not affect adult organisms, if they are grown under permissive conditions through this stage. As such, organisms may be distributed into the environment after the lethal life cycle stage, allowing the lethal system to be passed into the wild-type population through sexual reproduction. Other life cycle stages, such as the adult stage, may also be targeted by selection of genes or promoters expressed at specific life cycle stages, if appropriate.

Multiple copies

We prefer that the multicellular organism of the present invention has a copy of the lethal genetic system at more than one locus. Preferably, the lethal system is homozygous at more than one locus.

Multiple copies of the lethal system are useful to enhance the effect of the invention. For example, if the organism is homozygous at one locus for a female specific lethal system, any females that result from mating of the organism with wild type females will be killed. Male

offspring will survive, and carry one copy of the system. Only 50 % of the next (second) generation of male offspring will carry the lethal system.

The approach will clearly be more effective if more than 50% of this next (second) generation of male offspring were to inherit the lethal genetic system. There are several ways of achieving this. For example, if the lethal genetic system is homozygous at more than one, not tightly linked locus, e.g. on more than one chromosome, then the proportion of these males carrying the lethal genetic system will increase. Specifically, with the lethal genetic system homozygous at two unlinked loci, the first generation males will be heterozygous at both loci, 75% of the second generation males will carry at least one copy of the lethal genetic system. Correspondingly, under restrictive conditions all of the first generation and 75% of the second generation females will die.

Another way of achieving this effect is to use a segregation distortion/meiotic drive system. In the Drosophila SD system, the SD chromosome is preferentially inherited from males heterozygous for SD and a normal (+) SD-sensitive chromosome. SD/+ males transmit SD-bearing, to the virtual exclusion of +-bearing, homologues; as many as 99% of the functional sperm may carry SD. Segregation distortion/meiotic drive systems are known in a wide range of insect and non-insect species.

A third way of ensuring >50% inheritance of the lethal genetic system in the second generation is to link the lethal genetic system to insecticide resistance and use the insecticide to eliminate some or all of the second (and subsequent) generation progeny which do not carry the lethal genetic system and hence do not carry the linked resistance gene.

X chromosome

The lethal system may be located on any chromosome, either an autosome or sex chromosome. In species where sex is determined by the X or Y chromosome content and where elimination of the transgene from the gene pool is desired, then we prefer that the lethal system is located on the X chromosome. Consider the case in which the lethal system is specific for females. A male organism (XY) having the lethal system on the X chromosome mates in the

wild with a female wild type organism (XX). The male offspring must derive their Y chromosome from the recombinant male and their X chromosome from their mother. These males are viable and have no lethal gene. Female offspring must derive one X chromosome from the recombinant male and, thus, contain the lethal genetic system - they are killed. As such, the lethal system is eliminated from the gene pool, which may be preferable if this element is a transgene.

The present technology also allows a method to be developed for the selection of males or females *per se*, comprising producing a organism as described herein containing a conditional dominant lethal system, wherein the lethal effect of the lethal system is sex-specific. Sex selection is achieved by allowing expression of the lethal effect of the lethal system, to eliminate one sex. The individual male or female population may then be used for any desired purpose, not being limited to biological control.

The present invention also relates to a method of producing a recombinant multicellular organism for use in the present invention, wherein the organism is transformed with a vector containing a dominant lethal system, or a suitable sequence for site specific mutation. We prefer that all the required elements to control the expression of the dominant lethal gene are present on a single transformation construct (vector). In this way, only a single transformation step, and single transformation marker, are required. In addition, use of a single transformation construct helps prevent recombination of separate elements of the lethal genetic system.

Alternatively, multiple vectors may be used to transform the organism with the necessary elements of the lethal system, if necessary. It is also possible that control elements and enhancers used to control, for example, a transcription factor which acts on the lethal gene, may also interfere with the lethal gene expression itself. It may, therefore, be necessary to separate the components using silencer elements.

The effect of a promoter or enhancer upon a gene normally requires the elements to be present on the same stretch of DNA. However, the effect of a transcription factor may be exerted in *trans*, and may be located on, for example, a different chromosome. The invention is not limited to integration of the controlling elements on the same chromosome.

The construction of a recombinant multicellular organism may require use of a transformation system for the target species (the species which is to be controlled). The specific nature of the transformation system is not a critical feature of the invention, and transformation protocols for a number of, for example, insects are already known.

Vectors may be constructed using standard molecular biology techniques in bacteria such as *E. coli*. We prefer that the vector used for transformation contains a selectable marker, such as genes producing G418 resistance or hygromycin resistance. Alternative genes other than those related to antibiotic resistance characteristics, such as green fluorescent protein (GFP) may also be used. Expressed under the control of a suitable promoter, this protein can be visualised simply by illuminating with a suitable excitatory wavelength (e.g. blue) and observing the fluorescence. Such a marker would also allow easy identification of trapped insects in release-and-recapture experiments.

Other suitable markers for transformation are well known to the person skilled in the art. The present invention also relates to vectors used to produce recombinant multicellular organisms as described herein.

The invention also relates to organisms comprising a conditional dominant lethal genetic system for use in a combined method of sex separation and biological control, as herein defined.

The present invention will now be illustrated with respect to the following Examples, which are for illustrative purposes only and are not limiting upon the present invention.

EXAMPLES: BIOLOGICAL CONTROL IN A *DROSOPHILA* MODEL

Introduction

In one specific embodiment, a two-part system may be used to produce a conditional lethal effect. This system is based upon the repressor (tetR) of the transposon-1-derived

tetracycline (Tc) resistance operon of *E. coli*. The use of this repressor for repressible gene expression in eukaryotes has been developed by Manfred Gossen and Hermann Bujard (reviewed in Gossen, M., Bonin, A. and Bujard, H. "Control of gene activity in eukaryotic cells by prokaryotic regulatory elements" TIBS 18 471-475 1993). In this system, the tetR gene product is fused to the acidic domain of VP16, to create a highly efficient Tc-repressible transactivator (tTA).

The first part of the system is the tTA expressed under the control of a suitable promoter, and the second part is a dominant lethal gene expressed under the control of the tTA. Overall, this gives expression of the dominant lethal in a Tc-repressible fashion. When tetracycline is not available, the tTA activates the lethal gene. When tetracycline is present, it binds to the tTA and prevents activation of the lethal gene by tTA. This lethal system is under the control of a promoter of choice. One further level of control can be exerted by the choice of which Tc-analogue to use for repression: different analogues will have different half-lives in the insect leading to induction of the killer gene more or less promptly after the repressor is withdrawn from the diet.

We prefer that a non-bactericidal analogue should be used, so as not to encourage tetracycline resistance in environmental micro-organisms. Use of a non-bactericidal analogue is in any case essential for species such as tsetse fly, which have symbiotic bacteria essential for reproduction of the fly which are killed by antibiotics.

Even this one system may be varied to provide a flexible tool for population control. Greater flexibility may be achieved by combining two or more promoters or enhancers. For example, medfly control might use expression in the adult female (to prevent release of egg-laying females), and in early embryonic development (to prevent larval growth within the fruit). Since this means expression before the embryo starts to feed for itself, it would be important for growing the stock that a relatively stable Tc analogue is used, so that the embryos survive because of the maternal contribution of Tc. Larval expression could be also used as an alternative, but with greater damage to the fruit.

Use of the above system to control the lethal effect of the lethal gene is only one example of how an effect could be achieved, and there are numerous promoters, transactivators and lethal genes, for example, which could be used to achieve the desired effect.

In the above scenario expression at more than one stage may be required. This could be achieved by using two separate tTA constructs, or by combining stage-specific enhancers into a single construct. Appropriate promoters for stage-specific expression may be identified by subtractive hybridisation or other known methods.

Insertion of the lethal gene or system into the chromosome of the transgenic organism may be at any suitable point. It is not necessary to determine the location of the lethal gene on the chromosome. Even though inserted elements may respond to control elements in adjacent chromatin, this not an issue for the tRE-killer lines, where lines giving inappropriate expression will probably not survive.

The present invention has been exemplified in the model insect species *Drosophila melanogaster*. Though *D. melanogaster* is not an economically important pest, it is experimentally tractable. The tTA system in general has been demonstrated in Drosophila (Bello, B., Resendez-Perez, D., Gehring, W.J. 1998 "Spatial and temporal targeting of gene expression in Drosophila by means of a tetracycline-dependent transactivator system." Development 125:2193-2202). The Hsp26-tTA and tRE-lacZ used below, and some vectors [described below], came from this paper.

Components:

Transactivator component (promoter - tTA)

Hsp26-tTA: Heat shock protein 26-tTA. Low basal level, heat-shock inducible to higher level, not sex-specific.

Obtained from Bruno Bello (NIMR, London). As detailed in Bello et al. (1998) Development 125, 2193-2202. Hsp26 promoter region with a portion of the translated region (sequences from

-1917 to +490) was fused to a tTa coding region isolated as an EcoRI/BamHI fragment from pUHD 15-1.neo followed by the transcription termination sequence of the Hsp70 gene.

Act5C-tTA: Actin 5C-tTA. Strong, constitutive, ubiquitous promoter, not sex-specific.

The tTA coding region was excised as an EcoRI/PvuII fragment then end filled using T4 DNA polymerase. The p CaSpeR {Actin5C GFP}(Reichhart and Ferrandon, 1998 Green balancers. D. I. S. 81: 201--202) was digested with XbaI/BamHI to remove the GFP fragment then end filled using T4 polymerase. These two fragments were then ligated. The resulting clones were screened using a SmaI/EcoRV digest to select a clone of the correct orientation, placing the tTa coding region under the control of the Actin 5C promoter.

Stwl-tTA: Stonewall-tTa. Female-specific in embryos, but expressed later in both sexes.

The tTa coding region was excised from the plasmid pUHD 15-1.neo by digestion with EcoRI and PvuII. This fragment was then ligated into the vector pstwl^{+mCa} (Clark, K.A.and McKearin D.M. (1996), Development 122 (3): 937—950) digested with EcoR1/PvuII such that tTa was placed under the transcriptional control of 1.7kb of stwl promoter genomic DNA.

Sxl^{pe}-tTA: Sex lethal-tTA. Early promoter (PE) from Sxl. Thought to be expressed in early female embryos only.

The tTa coding region was excised from the plasmid pUHD 15-1.neo (Gossen M. and Bujard H. (1992); PNAS, 89, 5547-51) by digestion with EcoRI/PvuII. This fragment was then ligated into the 5-1 sxl^{pe}: bluescript (containing Sxl^{pe} sequences (Keyes LN, et al. (1992) Cell. 6; 68(5): 933-43)digested with EcoRI and EcoRV to create sxlpe tTa bluescript. A KpnI/NotI fragment containing the tTa coding region and sxlpe promoter was subcloned into the P element transformation vector pP {W8} (Klemenz et al., (1987) Nucleic Acids Res. 15:) 3947—3959) digested with KpnI/NotI to create p(sxl^{pe} tTa).

Yp3-tTA: Yolk protein 3-tTA. Female fat body enhancer (FBE) from yolk protein 3, with hsp70 minimal promoter. Expressed in female fat body in larvae and adults.

The tTa coding region was excised from the plasmid pUHD 15-1.neo by digestion with EcoRI and PvuII. This fragment was then cloned between the EcoRI/PvuII sites of the yp 3 expression construct p*FBE* (Bownes M, personal communication) such that it was under the transcriptional control of the Female Fat Body Enhancer (FBE) (Ronaldson E, et al. Genet Res. 1995 Aug; 66(1): 9-17.) and a minimal viral promoter.

tRE- responsive gene

tRe-lacZ: *E. coli lacZ* gene, encoding β-galactosidase. Used as reporter. Obtained from Bruno Bello (NIMR, London). As detailed in Bello et al. (1998) Development 125, 2193-2202. The heptameric repeat of the tet operator was isolated as a EcoRI/KpnI fragment from pUHC 13-3 (Gossen M. and Bujard H. (1992); PNAS, 89,5547-51) and cloned upstream of the P-lacZ fusion of the enhancer-test vector CPLZ (Wharton KA and Crews ST. (1994) Development. 120(12): 3563-9.). CPLZ contains the P element transposase promoter (up to –42 from cap site) and the N-terminal transposase sequence fused in-frame with lacZ and the polyadenylation signal of SV40.

WTP-2 (white-tetO-P promoter – vector containing tRe sequences)

Obtained from Bruno Bello (NIMR, London). As detailed in Bello et al. (1998) Development 125, 2193-2202. This P-element vector was constructed to express any gene under the control of a tetracycline-responsive promoter. It contains the vector backbone of CPLZ, the heptameric repeat of the tet operator, the P-element promoter and leader sequences from Carnegie 4 (Rubin GM and Spradling AC (1983) Nucleic Acids Res Sep 24; 11(18): 6341-51) and the polyadenylation signal of SV40.

WTP-3 (modified WTP-2)

The WTP-2 vector was modified by the addition of two complimentary short oligos *5' UAS ATG+* (AATTGCCACCATGGCTCATATGGAATTCAGATCTG) and *3' UAS ATG-* (GGCCGCAGATCTGAATTCCATATGAGCCATGGTGGGC) into the WTP-2 MCS. The oligos were allowed to anneal and ligated to WTP-2 digested with EcoRI/NotI. These oligos

introduced a consensus translation start and several additional cloning sites into the WTP-2 multiple cloning site (MCS).

tRe-EGFP. Encodes a mutant version of Green Fluorescent Protein (GFP), a jellyfish (*Aequoria*) gene encoding a fluorescent protein. The EGFP mutant has two amino acid changes, giving a brighter, more soluble protein. Used as a reporter. The *enhanced green fluorescent protein* (EGFP, a F64L, S65T mutant derivative of GFP) coding region (Craven et al. (1998) Gene 9; 221(1): 59-68) was isolated as a NcoI/EcoRI fragment from pUASLPGFP (Louise Parker and Luke Alphey, in preparation) then end filled with T4 polymerase. The WTP-3 vector was then digested with EcoRI and end filled with T4 polymerase and the fragments ligated together. A diagnostic digest using PvuII/BamHI, was then used to select a clone of the correct orientation.

tRe-Ras64B^{V12}. Mutant version of *Drosophila melanogaster* Ras64B, involved in cell signalling. Mutant is constitutively active, making it toxic to the cell if expressed at a high enough level. Toxicity is not sex-specific. The Ras64B^{V12} cDNA was cloned as an EcoRI/NotI fragment from the p {sevRas64B^{V12}} (Matsuo et al., 1997 Development 124(14): 2671—2680)), into WTP-2 digested with EcoRI/NotI.

tRe- Msl-1^{Mpu}. Mutant version of *Drosophila melanogaster* Msl-1. Msl-1 is a component of the sex determination pathway that is usually expressed only in males, being repressed in females by a product of the *Sex lethal* gene. Activity of mutant is independent of Sex lethal, making it toxic to females if expressed at a high enough level. Toxicity is therefore sex-specific.The *msl-1*^{MPU} cDNA was cloned as an EcoRI fragment from M1-ECTOPIC (Chang and Kuroda, (1998) Genetics 150(2): 699—709) into the WTP-2 vector digested with EcoRI. A diagnostic digest using HindIII/NotI, was then used to select a clone of the correct orientation, placing the *msl-1*^{MPU} cDNA under the control of the tRe sequences.

tRe- Msl-2^{Nopu}. Mutant version of *Drosophila melanogaster* Msl-2. Msl-2 is another component of the sex determination pathway that is usually expressed only in males, being repressed in females by a product of the *Sex lethal* gene. Activity of mutant is independent of Sex lethal, making it toxic to females if expressed at a high enough level. Toxicity is therefore sex-specific.

The msl-2 cDNA was cloned as a NotI/XbaI fragment from pM2 NOPU (Kelley et al., Cell 81; 867-877, 1995) and cloned into WTP-2 digested with NotI/XbaI.

EXAMPLE 1: SINGLE CHROMOSOME CROSSES

In "single chromosome crosses" at 25°C, ten to fifteen virgin females homozygous for the tTA construct and five to ten young males homozygous for the tRe construct were placed on food containing or lacking a tetracycline supplement. Their progeny were allowed to develop on this food.

Sxl^{pe}

Tetracycline conc. µg/ml	Female	Total	Male	Total
0	0^{A},0^{B},0^{C},0^{F},0,0,0,0	0	58,47,60,51,46,60,52,54	428
0.1	46,49,50,51,52,50,41,40	379	56,42,72,41,56,72,61,34	434
1	52,40,60,0,60,72,50,52	386	50,51,55,3,63,54,57,56	389
5	41,55,49,52,48,47,40,51	383	36,47,42,55,36,55,52,52	375

Sxlpe $tTa^{(A,B,C,F)}$ x tRe $Ras64B^{V12(B,C)}$

Format for data: the 8 numbers are the results from crosses using independent insertions of each element (to control for position effect). Here, 4 insertions of Sxl^{pe}-tTA (A, B, C, and F) were used and two of tRE-Ras64B^{V12} (B and C). The order of the data are: Sxl^{pe}-tTA $^{(A)}$ females with tRe-Ras64B$^{V12\,(B)}$ males, then SxlB x RasB, SxlC x RasB, SxlF x RasB, SxlA x RasC, SxlB x RasC, SxlC x RasC and finally SxlF x RasC. Data are presented in a similar fashion in the other tables

Tetracycline conc. µg/ml	Female	Total	Male	Total
0	0,0,0,0,0,0,0,0	0	59,57,62,51,73,69,57, 55	483
0.1	61,52,47,46,22,31,36,15	296	60,62,56,71,69,75,55, 72	520
1	59,57,63,59,31,21,15,21	326	47,56,49,62,63,67,71, 58	473
5	61,47,52,56,38,22,16,12	304	68,72,67,92,58,54,61, 63	535

Sxlpe $tTa^{(A,B,C,F)}$ x tRe Msl-$1^{Mpu(A,B)}$

Tetracycline conc. µg/ml	Female	Total	Male	Total
0	0,0,0,0,0,0,0,0,0,0,0,0	0	56,72,81,69,62,63,56, 47,82,57,55,61	761
0.1	79,56,47,42,51,61,52,52, 49,51,53,54	647	58,41,40,35,50,67,71, 39,52,62,40,70	562
1	42,45,56,48,52,61,57,54, 55,56,57,61	644	60,39,61,60,69,49,59, 38,64,69,71,35	674
5	58,61,52,53,54,61,29,31, 55,50,49,62	615	61,59,57,56,55,48,91, 63,54,50,81,67	742

Sxlpe $tTa^{(A,B,C,F)}$ x tRe Msl-$2^{Nopu(B,C,D)}$

Stwl

Tetracycline conc. µg/ml	Female	Total	Male	Total
0	0,0,0,0,0,0	0	0,0,0,0,0,0	0
0.1	36,62,71,41,49,58	317	43,442,63,35,68	315

1	58,37,58,41,55,58	307	47,70,51,51,39,70	328
5	36,38,56,43,34,64	271	57,71,68,53,44,42	335

Stwl $tTa^{(A,B,C)}$ x tRe $Ras64B^{V12(B,C)}$

Tetracycline conc. µg/ml	Female	Total	Male	Total
0	0,0,0,0,0,0	0	50,44,45,56,40,67	302
0.1	67,56,37,23,16,12	211	56,53,50,61,42,74	336
1	69,64,41,13,31,18	236	33,70,39,45,40,70	257
5	52,42,49,19,20,41	223	37,80,41,48,80	291

Stwl $tTa^{(A,B,C)}$ x tRe $Msl\text{-}1^{Mpu(A,B)}$

Tetracycline conc. µg/ml	Female	Total	Male	Total
0	0,0,0,0,0,0,0,0,0	0	38,53,47,68,38,70,52,60, 55	481
0.1	54,57,41,64,40,63,39, 42,36	436	59,58,49,73,48,69,45,47, 43	491
1	46,34,35,63,47,70,64, 39,41	439	55,40,40,71,50,72,74,46, 42	490
5	52,70,37,34,35,57,49, 50,50	434	54,71,41,42,41,66,56,55, 55	481

Stwl $tTa^{(A,B,C)}$ x tRe $Msl\text{-}2^{Nopu(B,C,D)}$

Actin5C

Tetracycline conc. µg/ml	Female	Total	Male	Total
0	0,0,0,0,0,0	0	0,0,0,0,0,0	377
0.1	77,57,69,50,45,63	361	50,70,71,67,53,61	372
1	86,59,60,80,70,72	427	46,89,72,45,76,55	383
5	46,49,87,63,59,71	375	75,83,58,83,72,82	400

Actin5C $tTa^{(B,C,E)}$ x tRe $Ras64B^{V12(B,C)}$

Tetracycline conc. µg/ml	Female	Total	Male	Total
0	0,0,0,0,0,0	0	83,73,65,69,53,80	423
0.1	72,74,80,68,72,46	412	82,52,57,66,86,59	402
1	61,83,48,66,65,57	321	74,69,85,58,48,61	351
5	70,57,50,62,61,86	386	48,68,52,62,84,87	401

Actin5C $tTa^{(B,C,E)}$ x tRe $Msl\text{-}1^{Mpu(A,B)}$

Tetracycline conc. µg/ml	Female	Total	Male	Total
0	0,0,0,0,0,0,0,0,0	0	63,52,67,71,88,55,46,86, 75	603
0.1	84,85,83,73,48,48,46,71, 58	548	62,54,48,81,85,74,78,77, 78	637
1	70,70,66,81,50,52,69,81, 51	590	69,87,47,64,66,59,58,47, 52	549
5	67,70,87,61,54,54,67,74, 81	615	71,61,57,53,51,65,45,68, 51	522

Actin5C $tTa^{(B, C, E)}$ x tRe $Msl\text{-}2^{Nopu (B, C, D)}$

Hsp26

Tetracycline conc. µg/ml	Female	Total	Male	Total
0	0,0,0,0	0	0,0,0,0	0
0.1	47,56,71,61	235	46,52,53,59	210
1	60,46,52,41	199	79,71,68,56	274
5	2,51,71,32	156	0,49,62,43	154

Hsp26 $tTa^{(A)}$ x tRe $Ras64B^{V12(B,C,)}$

Tetracycline conc. µg/ml	Female	Total	Male	Total
0	0,0,0,0,0,0	0	64,58,33,66,55,42	318
0.1	45,44,72,56,62,49	328	53,54,80,57,66,58	368
1	70,35,61,50,57,37	310	78,36,70,56,61,42	343
5	44,58,58,59,42,52	313	46,68,66,64,48,55	347

Hsp26 $tTa^{(A)}$ x tRe $Msl\text{-}1^{Mpu(A,B,)}$

Tetracycline conc. µg/ml	Female	Total	Male	Total
0	0,0,0	0	56,47,56	159
0.1	48,49,62	159	56,68,49	159
1	43,45,51	135	36,39,47	122
5	55,3,66	124	61,5,54	120

Hsp26 $tTa^{(A)}$ x tRe $Msl\text{-}2^{Nopu(A,B,)}$

Yp3

Tetracycline conc. µg/ml	Female	Total	Male	Total
0	0,0,0,0,0,0	0	65,70,61,65,47,42	350
0.1	33,54,50,72,63,50	322	42,64,52,74,67,54	352
1	56,56,61,69,57,43	342	59,64,65,75,64,49	376
5	46,51,73,65,42,39	316	44,56,79,74,52,49	354

Yp3 $tTa^{(A)}$ x tRe $Ras64B^{V12(B,C,)}$

Tetracycline conc. µg/ml	Female	Total	Male	Total
0	0,0,2,0,0,0	2	49,58,39,65,35,51	297
0.1	36,65,71,37,59,68	336	46,73,77,46,66,71	379
1	42,65,67,57,35,53	319	49,72,68,59,41,58	347
5	55,55,43,58,36,60	307	63,64,49,63,45,64	348

Yp3 $tTa^{(A)}$ x tRe $Msl\text{-}1^{Mpu(A,B,)}$

Tetracycline conc. µg/ml	Female	Total	Male	Total
0	0,0,0,0,0,0	0	35,35,72,52,45,37	276
0.1	34,68,42,51,33,40	268	35,72,45,56,36,44	248
1	41,39,42,60,70,72	324	51,49,46,61,78,77	362
5	70,55,56,65,43,61	349	74,58,64,73,51,66	386

Yp3 $tTa^{(A)}$ x tRe $Msl\text{-}2^{Nopu(A,B,)}$

Conclusion

These data show that one or both sexes can be efficiently eliminated, while good repression of this lethality can be achieved by the addition of modest concentrations of tetracycline to the food. This repression is effective over a wide range of tetracycline concentrations.

EXAMPLE 2: REPORTER CROSSES

In "reporter crosses" at 25°C, females homozygous carrying an insertion of $Sxlp^{e}$ tTa on their X chromosome ($Sxlp^{e}$ $tTa^{(A)}$) were crossed to males carrying various reporter constructs. As with "single chromosome crosses", ten to fifteen virgin females homozygous for the tTA construct and five to ten young males homozygous for the tRe construct were placed on food containing or lacking a tetracycline supplement. Their progeny were allowed to develop on this food.

lacZ

Embryos were stained for lacZ using a standard histochemical method.

Tetracycline conc. µg/ml	LacZ positive	Total	LacZ negative	Total
0	60,85,99,60	304	78,89,85,93	345
0.1	0,0,0,0	0	176,174,178,181	709
1	0,0,0,0	0	188,190,181,180	739
5	0,0,0,0	0	156,151,159,185	651

(Female) $Sxlp^{e}$ $tTa^{(A)}$ x tRe $lacZ^{(III)}$ (Male)

Tetracycline conc. µg/ml	LacZ positive	Total	LacZ negative	Total
0	57,82,97,45	281	61,74,59,82	276
0.1	0,0,0,0	0	131,165,132,90	518
1	0,0,0,0	0	170,161,181,195	707
5	0,0,0,0	0	126,190,190,196	702

(Male) $Sxlp^{e}$ $tTa^{(A)}$ x tRe $lacZ^{(III)}$ (Female)

Tetracycline conc. µg/ml	LacZ positive	Total	LacZ negative	Total
0	0,0,0,0	0	189,200,153,169	711
0.1	0,0,0,0	0	164,175,190,179	708
1	0,0,0,0	0	182,190,195,167	737
5	0,0,0,0	0	199,151,169,164	683

(Male) Sxlp^{e} tTa$^{(A)}$ tRe lacZ$^{(I)}$ x C(1)DX (Female)

EGFP

Embryos were scored for fluorescence. In the case of embryos on tetracycline-free media, these were separated, allowed to develop on tetracycline-free media and the sex of the emerging adults was scored.

Tetracycline conc. µg/ml	Fluorescent	female	male	Non-Fluorescent	female	male
0	89,100,53,55	200	0	99,86,46,51	0	232
0.1	0,0,0,0	-	-	199,182,188, 153	-	-
1	0,0,0,0	-	-	170,135,163, 196	-	-
5	0,0,0,0	-	-	186,159,127, 200	-	-

(Female) Sxlp^{e} tTa$^{(A)}$ x tRe EGFP$^{(II)}$ (Male)

Tetracycline conc. µg/ml	Fluorescent	female	male	Non-Fluorescent	female	male
0	60,91,62,83	243	0	102,56,79,72	1	256
0.1	0,0,0,0	-	-	196,170,165, 162	-	-
1	0,0,0,0	-	-	182,200,197, 161	-	-
5	0,0,0,0	-	-	182,161,188, 182	-	-

(Male) $Sxlp^{e}$ $tTa^{(A)}$ x tRe $EGFP^{(II)}$ (Female)

Tetracycline conc. µg/ml	Fluorescent	male	female	Non-Fluorescent	male	female
0	0,0,0,0	-	-	196,179,165, 164	-	-
0.1	0,0,0,0	-	-	179,197,198, 188	-	-
1	0,0,0,0	-	-	198,187,190, 164	-	-
5	0,0,0,0	-	-	170,177,199, 165	-	-

(Male) $Sxlp^{e}$ $tTa^{(A)}$; tRe $EGFP^{(II)}$ x C(1)DX (Female)

C(1)DX is a compound X chromosome; effectively two X chromosomes joined together. The X chromosome from males crossed to C(1)DX females is therefore inherited by the sons, rather than the daughters.

Conclusions

The data demonstrate that, as expected, reporter gene expression is turned off in the presence of tetracycline over a range of concentrations.

EXAMPLE 3: RECOMBINANT CHROMOSOME EXPERIMENTS

40-45 young females and 20-25 young males raised at 25°C upon food with the indicated tetracycline supplement were allowed to mate, then transferred to normal (tetracycline-free) food after 3-4 days. These flies were transferred to fresh vials of normal food every day for 12 days, then removed on the 13th day. All the vials were incubated at 25°C while the progeny developed. The numbers of male and female progeny emerging as adults in each vial were recorded.

Tetracycline concentration

Sxl^{Pe}

Tet. Conc.	Day 1		Day 2		Day 3		Day 4		Day 5		Day 6		Day 7	
µg/ml	Male	Female	Male	Female	Male	Female	Male	Female	Male	Female	Male	Female	Male	Female
0.1	103	0	98	0	89	0	92	0	105	0	95	0	110	0
1	128	0	137	0	150	0	136	0	111	0	87	0	100	0
5	110	0	111	0	95	0	90	0	144	0	93	0	138	0
20	131	0	126	0	133	0	120	0	93	0	99	0	111	0
100	139	0	127	0	145	0	110	0	149	0	128	0	94	0
500	95	11	133	12	145	1	137	1	86	0	112	0	128	0
1000	140	12	133	24	119	8	94	2	92	1	137	1	129	1
2000	110	35	97	25	94	16	138	12	115	2	126	1	145	1

Tet. Conc.	Day 8		Day 9		Day 10		Day 11		Day 12		Total	
µg/ml	Male	Female	Male	Female	Male	Female	Male	Female	Male	Female	Male	Female
0.1	106	0	131	0	148	0	86	0	99	0	1262	0
1	106	0	109	0	97	0	124	0	114	0	1399	0
5	106	0	89	0	148	0	148	0	87	0	1359	0
20	87	0	149	0	104	0	113	0	132	0	1398	0
100	93	0	125	0	99	0	121	0	139	0	1469	0
500	142	0	129	0	114	0	131	0	126	0	1478	25
1000	89	0	94	0	97	0	138	0	87	0	1349	49
2000	94	0	137	0	99	0	141	0	143	0	1439	92

Sxl^{Pe} -tTA, tRE-$Ras64B^{V12}$ on the X chromosome.

Tet. Conc.	Day 1		Day 2		Day 3		Day 4		Day 5		Day 6		Day 7	
µg/ml	Male	Female	Male	Female	Male	Female	Male	Female	Male	Female	Male	Female	Male	Female
0.1	103	0	98	0	149	0	121	0	134	0	150	0	117	0
1	149	0	86	0	111	0	112	0	126	0	148	0	136	0

Tet. Conc.	Day 1		Day 2		Day 3		Day 4		Day 5		Day 6		Day 7	
µg/ml	Male	Female	Male	Female	Male	Female	Male	Female	Male	Female	Male	Female	Male	Female
5	104	0	99	0	148	0	128	0	142	0	134	0	93	0
20	121	0	106	0	97	0	127	0	142	0	131	0	107	0
100	94	0	142	0	115	0	131	0	114	0	103	0	131	0
500	140	34	148	23	100	14	95	1	122	0	120	0	115	0
1000	110	29	87	12	138	22	145	17	91	5	106	1	102	1
2000	123	42	145	37	131	43	139	15	126	12	118	7	100	4

Tet. Conc.	Day 8		Day 9		Day 10		Day 11		Day 12		Total	
µg/ml	Male	Female	Male	Female	Male	Female	Male	Female	Male	Female	Male	Female
0.1	138	0	142	0	147	0	130	0	112	0	1541	0
1	129	0	123	0	91	0	99	0	131	0	1441	0
5	99	0	106	0	95	0	144	0	129	0	1421	0
20	149	0	150	0	89	0	128	0	140	0	1487	0
100	93	0	119	0	143	0	87	0	144	0	1416	0
500	98	0	129	0	90	0	124	0	107	0	1388	72
1000	92	0	150	0	145	0	107	0	143	0	1416	87
2000	92	1	120	0	89	0	106	0	149	0	1438	161

Sxl^{pe} -tTA, tRE-$Ras64B^{V12}$ on the third chromosome.

Tet. Conc.	Day 1		Day 2		Day 3		Day 4		Day 5		Day 6		Day 7	
µg/ml	Male	Female	Male	Female	Male	Female	Male	Female	Male	Female	Male	Female	Male	Female
0.1	97	0	136	0	152	0	130	0	108	0	114	0	88	0
1	102	0	99	0	134	0	171	0	171	0	118	0	91	0
5	130	0	159	0	156	0	91	0	84	0	127	0	110	0
20	76	0	129	0	126	0	79	0	89	0	98	0	94	0
100	112	0	145	0	130	0	124	0	79	0	109	0	134	0
500	136	2	79	0	161	0	102	0	171	0	151	0	161	0
1000	92	15	83	9	150	3	149	2	146	0	92	0	115	0
2000	127	21	95	14	153	3	164	4	135	1	97	1	144	0

Tet. Conc.	Day 8		Day 9		Day 10		Day 11		Day 12		Total	
µg/ml	Male	Female	Male	Female	Male	Female	Male	Female	Male	Female	Male	Female
0.1	140	0	104	0	141	0	173	0	81	0	1464	0
1	104	0	120	0	171	0	102	0	144	0	1527	0
5	116	0	123	0	155	0	163	0	121	0	1535	0
20	122	0	103	0	126	0	123	0	78	0	1243	0
100	127	0	133	0	79	0	157	0	154	0	1483	0
500	164	0	95	0	160	0	154	0	91	0	1625	2
1000	168	0	153	0	80	0	95	0	79	0	1402	29
2000	158	0	103	0	129	0	141	0	97	0	1543	44

Sxl^{pe} -tTA, tRE-$Msl\text{-}2^{Nopu}$ on the X chromosome.

Tet. Conc.	Day 1		Day 2		Day 3		Day 4		Day 5		Day 6		Day 7	
µg/ml	Male	Female	Male	Female	Male	Female	Male	Female	Male	Female	Male	Female	Male	Female
0.1	111	0	108	0	130	0	69	0	101	0	110	0	130	0
1	89	0	106	0	119	0	70	0	87	0	117	0	138	0
5	112	0	80	0	68	0	130	0	78	0	93	0	78	0
20	92	0	83	0	129	0	127	0	66	0	69	0	95	0
100	72	0	90	0	72	0	66	0	106	0	122	0	100	0
500	78	0	118	0	69	0	67	0	88	0	83	0	135	0
1000	122	2	107	1	133	0	116	0	115	0	107	0	119	0
2000	134	12	79	14	123	5	130	1	102	0	114	0	83	0

Tet. Conc.	Day 8		Day 9		Day 10		Day 11		Day 12		Total	
µg/ml	Male	Female	Male	Female	Male	Female	Male	Female	Male	Female	Male	Female
0.1	127	0	92	0	79	0	77	0	133	0	1267	0
1	71	0	104	0	81	0	124	0	65	0	1171	0
5	106	0	84	0	135	0	119	0	82	0	1165	0
20	101	0	71	0	108	0	74	0	112	0	1127	0
100	136	0	104	0	116	0	77	0	107	0	1168	0
500	128	0	104	0	73	0	106	0	88	0	1137	0
1000	101	0	115	0	86	0	96	0	92	0	1309	3
2000	130	0	105	0	120	0	104	0	101	0	1325	32

Sxl^{Pe} -tTA, tRE-Msl-2^{Nopu} on the third chromosome.

Tet. Conc.	Day 1		Day 2		Day 3		Day 4		Day 5		Day 6		Day 7	
µg/ml	Male	Female	Male	Female	Male	Female	Male	Female	Male	Female	Male	Female	Male	Female
0.1	93	0	137	0	84	0	66	0	114	0	107	0	114	0
1	73	0	90	0	99	0	120	0	118	0	85	0	85	0
5	84	0	122	0	131	0	93	0	104	0	90	0	133	0
20	127	0	128	0	80	0	105	0	81	0	122	0	108	0
100	72	0	80	0	87	0	128	0	78	0	92	0	86	0
500	98	0	78	0	94	1	105	0	138	0	77	0	92	0
1000	117	1	105	2	130	1	130	1	82	0	88	0	113	0
2000	91	16	70	11	69	13	70	4	108	1	90	0	115	0

Tet. Conc.	Day 8		Day 9		Day 10		Day 11		Day 12		Total	
µg/ml	Male	Female	Male	Female	Male	Female	Male	Female	Male	Female	Male	Female
0.1	117	0	78	0	123	0	125	0	121	0	1279	0
1	91	0	90	0	68	0	88	0	82	0	1089	0
5	89	0	70	0	138	0	85	0	100	0	1239	0
20	95	0	118	0	70	0	114	0	114	0	1262	0
100	66	0	137	0	85	0	109	0	93	0	1113	0
500	68	0	70	0	109	0	86	0	136	0	1151	1
1000	95	0	137	0	99	0	120	0	66	0	1282	5

2000	84	0	98	0	83	0	128	0	131	0	1137	45

Sxl^{pe} -tTA, tRE-Msl-1^{Mpu} on the X chromosome.

Hsp26

Tet. Conc.	Day 1		Day 2		Day 3		Day 4		Day 5		Day 6		Day 7	
µg/ml	Male	Female	Male	Female	Male	Female	Male	Female	Male	Female	Male	Female	Male	Female
0.1	153	0	154	0	127	0	130	0	81	0	151	0	147	0
1	138	0	98	0	74	0	88	0	150	0	123	0	115	0
5	140	0	132	0	119	0	129	0	87	0	156	0	157	0
20	115	0	113	0	92	0	92	0	129	0	77	0	119	0
100	150	0	127	0	126	0	114	0	78	0	93	0	98	0
500	119	1	146	0	154	0	132	0	112	0	97	0	80	0
1000	77	5	109	2	105	2	85	0	84	0	127	0	91	0
2000	156	18	101	6	149	3	115	1	134	0	139	0	151	0

Tet. Conc.	Day 8		Day 9		Day 10		Day 11		Day 12		Total	
µg/ml	Male	Female	Male	Female	Male	Female	Male	Female	Male	Female	Male	Female
0.1	117	0	81	0	106	0	135	0	141	0	1523	0
1	152	0	89	0	105	0	146	0	89	0	1367	0
5	79	0	148	0	120	0	92	0	119	0	1478	0
20	69	0	78	0	149	0	72	0	116	0	1221	0
100	121	0	126	0	157	0	141	0	143	0	1474	0
500	142	0	103	0	104	0	144	0	129	0	1462	1
1000	75	0	147	0	105	0	97	0	123	0	1225	9
2000	86	0	97	0	98	0	131	0	76	0	1433	28

Hsp26-tTA, tRE-Msl-2^{Nopu} on the second chromosome.

Tet. Conc.	Day 1		Day 2		Day 3		Day 4		Day 5		Day 6		Day 7	
µg/ml	Male	Female	Male	Female	Male	Female	Male	Female	Male	Female	Male	Female	Male	Female
0.1	120	0	87	0	127	0	115	0	121	0	80	0	100	0
1	84	0	153	0	100	0	88	0	93	0	71	0	126	0
5	134	0	95	0	122	0	141	0	80	0	77	0	106	0
20	135	0	137	0	140	0	135	0	107	0	141	0	89	0
100	146	1	146	0	82	0	106	0	118	0	118	0	82	0
500	124	12	144	8	99	2	154	1	137	0	96	1	75	0
1000	72	27	85	17	76	15	87	12	102	5	93	5	69	3
2000	132	67	96	45	119	38	135	35	104	22	90	17	149	12

Tet. Conc.	Day 8		Day 9		Day 10		Day 11		Day 12		Total	
µg/ml	Male	Female	Male	Female	Male	Female	Male	Female	Male	Female	Male	Female
0.1	151	0	79	0	108	0	69	0	107	0	1264	0

1	78	0	95	0	105	0	112	0	154	0	1259	0
5	135	0	84	0	152	0	145	0	142	0	1413	0
20	79	0	157	0	92	0	73	0	139	0	1424	0
100	96	0	135	0	86	0	106	0	157	0	1378	1
500	139	0	142	0	145	0	84	0	136	0	1475	24
1000	114	1	145	0	130	0	136	0	152	0	1261	85
2000	149	2	81	0	127	0	146	0	88	0	1416	238

Hsp26-tTA, tRE-Msl-1^{Mpu} on the second chromosome.

Yp3

Tet. Conc.	Day 1		Day 2		Day 3		Day 4		Day 5		Day 6		Day 7	
µg/ml	Male	Female	Male	Female	Male	Female	Male	Female	Male	Female	Male	Female	Male	Female
0.1	93	0	119	0	112	0	141	0	100	0	126	0	89	0
1	117	0	135	0	122	0	121	0	127	0	101	0	136	0
5	112	0	116	0	128	0	111	0	136	0	113	0	130	0
20	89	0	107	0	107	0	98	0	88	0	102	0	107	0
100	129	0	136	0	128	0	127	0	135	0	144	0	107	0
500	136	2	88	0	113	0	113	0	87	0	94	0	109	0
1000	107	13	140	5	110	0	141	0	98	0	129	0	88	0
2000	119	32	102	15	107	12	109	9	109	8	140	2	127	0

Tet. Conc.	Day 8		Day 9		Day 10		Day 11		Day 12		Total	
µg/ml	Male	Female	Male	Female	Male	Female	Male	Female	Male	Female	Male	Female
0.1	105	0	133	0	93	0	131	0	121	0	1363	0
1	90	0	119	0	94	0	98	0	100	0	1360	0
5	119	0	96	0	88	0	144	0	91	0	1384	0
20	135	0	126	0	143	0	123	0	141	0	1366	0
100	96	0	92	0	104	0	94	0	115	0	1407	0
500	141	0	144	0	123	0	104	0	124	0	1376	2
1000	138	0	105	0	124	0	115	0	114	0	1409	18
2000	114	0	123	0	132	0	115	0	107	0	1404	78

Yp3-tTA, tRE-Ras64B^{V12} on the second chromosome.

Tet. Conc.	Day 1		Day 2		Day 3		Day 4		Day 5		Day 6		Day 7	
µg/ml	Male	Female	Male	Female	Male	Female	Male	Female	Male	Female	Male	Female	Male	Female
0.1	121	0	94	0	103	0	93	0	96	0	119	0	119	0
1	95	0	123	0	79	0	78	0	130	0	103	0	112	0
5	109	0	110	0	118	0	124	0	86	0	122	0	90	0
20	81	0	89	0	127	0	82	0	81	0	79	0	128	0
100	112	0	87	1	87	1	113	0	95	1	91	1	84	1
500	84	21	96	16	86	15	124	9	123	5	86	3	106	1
1000	100	47	110	12	109	8	103	13	102	9	97	2	82	6
2000	127	63	130	54	128	34	117	21	89	12	87	11	90	4

Tet. Conc.	Day 8		Day 9		Day 10		Day 11		Day 12		Total	
µg/ml	Male	Female	Male	Female	Male	Female	Male	Female	Male	Female	Male	Female
0.1	84	0	127	0	104	0	76	0	95	0	1231	0
1	94	0	106	0	83	0	93	0	113	0	1209	0
5	132	0	126	0	76	0	128	0	102	0	1323	0
20	119	0	99	0	90	0	106	0	87	0	1168	0
100	85	1	122	0	114	0	90	0	126	0	1206	6
500	85	1	93	0	111	0	111	0	104	0	1209	71
1000	95	0	113	0	110	0	85	0	87	0	1193	97
2000	131	1	128	0	91	0	95	0	82	0	1295	200

Yp3-tTA, tRE-Msl-2^{Nopu} on the second chromosome.

Tet. Conc.	Day 1		Day 2		Day 3		Day 4		Day 5		Day 6		Day 7	
µg/ml	Male	Female	Male	Female	Male	Female	Male	Female	Male	Female	Male	Female	Male	Female
0.1	91	0	84	0	107	0	80	0	88	0	92	0	99	0
1	117	0	92	0	128	0	80	0	104	0	116	2	8	0
5	82	0	123	0	116	0	120	2	89	0	90	0	95	0
20	92	1	101	0	87	0	109	0	81	0	121	0	83	1
100	108	13	130	9	131	5	99	7	109	3	123	1	107	1
500	78	22	85	16	80	12	106	15	130	11	91	10	118	7
1000	130	35	86	42	78	26	116	14	80	12	82	17	77	15
2000	116	79	130	72	78	44	101	29	132	32	94	22	89	16

Tet. Conc.	Day 8		Day 9		Day 10		Day 11		Day 12		Total	
µg/ml	Male	Female	Male	Female	Male	Female	Male	Female	Male	Female	Male	Female
0.1	88	1	135	0	123	0	128	0	114	0	1229	1
1	101	0	127	2	84	0	101	0	79	0	1137	4
5	80	0	94	0	127	0	128	3	86	0	1230	5
20	132	0	81	0	88	0	112	0	127	0	1214	2
100	106	1	132	0	81	0	115	0	107	0	1348	40
500	115	2	98	0	86	0	82	4	115	0	1184	99
1000	131	3	104	1	99	0	125	0	108	0	1216	165
2000	91	8	88	2	85	5	114	0	80	0	1198	309

Yp3-tTA, tRE-Msl-1^{Mpu} on the second chromosome.

Conclusions

These data show that feeding the mothers high concentrations of tetracycline has some protective effect, but that all these recombinant chromosomes work extremely efficiently over a

wide range of (parental) tetracycline concentrations, with the sole exception of "Yp3 tTa, tRe Msl-1^{Mpu} on the 2nd chromosome", which has some (<1%) escapers even at low tetracycline concentrations. Since there is no meiotic recombination in *Drosophila melanogaster* males, any of these recombinant chromosomes could be used in a genetic sexing or insect control program, if required. In practice, *Drosophila melanogaster* is not an agricultural pest or disease vector, but these data demonstrate that the effective elimination of one sex can be achieved by this method.

EXAMPLE 4: USE OF NON-ANTIBIOTIC TETRACYCLINE ANALOGUES

Recombinant chromosome stocks can readily be maintained at 25°C on epioxytetracycline concentrations of 1 μg/ml or anhydrotetracycline concentrations of 0.1 μg/ml, showing that these non-antibiotic tetracycline analogues are effective in repressing tTA responsive gene expression.

Epioxytetracycline

A standard range of additive concentrations were used in the following experiments (0.05 - 20 μg/ml). We were unable to maintain stock at two lowest concentrations, so marked n.d. (= "not done")

Epioxytetracycline Conc. μg/ml	Female	Male
0.05	n.d.	n.d.
0.1	n.d.	n.d.
1	0	1306
5	0	1581
20	0	1495

Sxlp^{e} tTa, tRe Ras64B^{V12} on the X chromosome

Epioxytetracycline Conc. µg/ml	Female	Male
0.05	n.d.	n.d.
0.1	n.d.	n.d.
1	0	1165
5	0	1279
20	0	1257

Sxlp^{e} tTa, tRe Ras64B^{V12} on the 3rd chromosome

Epioxytetracycline Conc. µg/ml	Female	Male
0.05	n.d.	n.d.
0.1	n.d.	n.d.
1	0	1076
5	0	1119
20	0	1159

Sxlp^{e} tTa, tRe Msl-2^{Nopu} on the X chromosome

Epioxytetracycline Conc. µg/ml	Female	Male
0.05	n.d.	n.d.
0.1	n.d.	n.d.
1	0	1250
5	0	1300
20	0	1364

Sxlp^{e} tTa, tRe Msl-2^{Nopu} on the 3rd chromosome

Epioxytetracycline Conc. µg/ml	Female	Male
0.05	n.d.	n.d.
0.1	n.d.	n.d.
1	0	1483
5	0	1585
20	0	1565

Sxlp^{e} tTa , tRe Msl-1^{Mpu} on the X chromosome

Epioxytetracycline Conc. µg/ml	Female	Male
0.05	n.d.	n.d.
0.1	n.d.	n.d.
1	0	1362
5	0	1181
20	0	1403

Hsp26 tTa , tRe Msl-2^{Nopu} on the 2nd chromosome

Epioxytetracycline Conc. µg/ml	Female	Male
0.05	n.d.	n.d.
0.1	n.d.	n.d.
1	0	1243
5	0	1409
20	0	1373

Hsp26 tTa , tRe Msl-1^{Mpu} on the 2nd chromosome

Epioxytetracycline Conc. µg/ml	Female	Male
0.05	n.d.	n.d.
0.1	n.d.	n.d.
1	0	1431
5	0	1424
20	0	1387

Yp3 tTa, tRe Ras64B^{V12} on the 2nd chromosome

Epioxytetracycline Conc. µg/ml	Female	Male
0.05	n.d.	n.d.
0.1	n.d.	n.d.
1	0	1350
5	0	1308
20	0	1343

Yp3 tTa, tRe Msl-1^{Mpu} on the X chromosome

Anhydrotetracycline

Anhydrotetracycline Conc. µg/ml	Female	Male
0.05	0	1452
0.1	0	1528
1	0	1614
5	0	1448
20	5	1592

Sxlp^{e} tTa, tRe Ras64B^{V12} on the X chromosome

Anhydrotetracycline Conc. µg/ml	Female	Male
0.05	0	1381
0.1	0	1304
1	0	1121
5	0	1269
20	1	1247

$Sxlp^{e}$ tTa, tRe $Ras64B^{V12}$ on the 3rd chromosome

Anhydrotetracycline Conc. µg/ml	Female	Male
0.05	0	1114
0.1	0	1120
1	0	1130
5	0	1148
20	0	1128

$Sxlp^{e}$ tTa, tRe $Msl\text{-}2^{Nopu}$ on the X chromosome

Anhydrotetracycline Conc. µg/ml	Female	Male
0.05	0	1331
0.1	0	1431
1	0	1309
5	0	1359
20	1	1362

$Sxlp^{e}$ tTa, tRe $Msl\text{-}2^{Nopu}$ on the 3rd chromosome

Anhydrotetracycline Conc. µg/ml	Female	Male
0.05	0	1582
0.1	0	1499
1	0	1474
5	0	1619
20	5	1533

Sxlp^{e} tTa, tRe Msl-1^{Mpu} on the X chromosome

Anhydrotetracycline Conc. µg/ml	Female	Male
0.05	0	707
0.1	0	1457
1	0	1437
5	0	773
20	5	1447

Hsp26 tTa, tRe Msl-2^{Nopu} on the 2nd chromosome

Anhydrotetracycline Conc. µg/ml	Female	Male
0.05	0	1492
0.1	0	1426
1	0	1418
5	0	1457
20	8	1499

Hsp26 tTa, tRe Msl-1^{Mpu} on the 2nd chromosome

Anhydrotetracycline Conc. µg/ml	Female	Male
0.05	0	1449
0.1	0	1411
1	0	1397
5	0	1430
20	2	1428

Yp3 tTa, tRe Ras64B^{V12} on the 2nd chromosome

Anhydrotetracycline Conc. µg/ml	Female	Male
0.05	0	1339
0.1	0	1263
1	0	1265
5	0	1284
20	0	1297

Yp3 tTa, tRe Msl-1^{Mpu} on the X chromosome

Anhydrotetracycline Conc. µg/ml	Female	Male
0.05	0	1316
0.1	0	1358
1	0	1354
5	0	1344
20	1	1312

Yp3 tTa, tRe Msl-2^{Nopu} on the 2nd chromosome

Conclusions

These data show that non-antibiotic analogues of tetracycline analogues can be used in place of tetracycline. In the case of epioxytetracycline, slightly higher concentrations are required to

repress gene expression. Neither has parental transmission characteristics substantially different from tetracycline, allowing for the different effective concentrations.

EXAMPLE 5 EFFECT OF TEMPERATURE

All the preceding experiments were performed at 25°C, the standard temperature for Drosophila culture. However, the insects in the wild would clearly be exposed to varying temperatures, so we investigated the extent to which the efficiency of the system is affected by temperature. As with the recombinant chromosome experiments, 40-45 young virgin females and 20-25 young males raised at 25°C upon food with the indicated tetracycline supplement were allowed to mate, then transferred to normal (tetracycline-free) food after 3-4 days. These flies were transferred to fresh vials of normal food every day. The numbers of male and female progeny emerging as adults in each vial were recorded. These experiments were performed at either 18°C or 29°C.

18°C

Tetracycline Conc. µg/ml	Female	Male
0.1	8	982
1	10	912
5	7	871

$Sxlp^{e}$ tTa, tRe $Ras64B^{V12}$ on the X chromosome

Tetracycline Conc. µg/ml	Female	Male
0.1	6	1065
1	9	1124
5	7	989

$Sxlp^{e}$ tTa, tRe $Ras64B^{V12}$ on the 3^{rd} chromosome

Tetracycline Conc. µg/ml	Female	Male
0.1	6	695
1	8	816
5	8	785

$Sxlp^{e}$ tTa, tRe $Msl\text{-}2^{Nopu}$ on the X chromosome

Tetracycline Conc. µg/ml	Female	Male
0.1	2	973
1	9	985
5	5	983

$Sxlp^{e}$ tTa, tRe $Msl\text{-}2^{Nopu}$ on the 3^{rd} chromosome

Tetracycline Conc. µg/ml	Female	Male
0.1	8	840
1	5	927
5	8	837

$Sxlp^{e}$ tTa, tRe $Msl\text{-}1^{Mpu}$ on the X chromosome

Tetracycline Conc. µg/ml	Female	Male
0.1	8	832
1	7	879
5	4	818

Hsp26 tTa, tRe $Msl\text{-}2^{Nopu}$ on the 2^{nd} chromosome

Tetracycline Conc. µg/ml	Female	Male
0.1	6	628
1	3	614
5	5	712

Hsp26 tTa, tRe $Msl\text{-}1^{Mpu}$ on the 2^{nd} chromosome

Tetracycline Conc. µg/ml	Female	Male
0.1	8	1152
1	12	1122
5	3	1225

Yp3 tTa, tRe Msl-2^{Nopu} on the 2^{nd} chromosome

Tetracycline Conc. µg/ml	Female	Male
0.1	5	1303
1	14	1218
5	7	1386

Yp3 tTa, tRe Msl-1^{Mpu} on the X chromosome

Tetracycline Conc. µg/ml	Female	Male
0.1	2	1190
1	4	1213
5	0	1058

Yp3 tTa, tRe Ras64B^{V12} on the 2^{nd} chromosome

29°C

Tetracycline Conc. µg/ml	Female	Male
0.1	0	716
1	0	711
5	0	715

Sxlp^{e} tTa, tRe Ras64B^{V12} on the X chromosome

Tetracycline Conc. µg/ml	Female	Male
0.1	0	781
1	0	749
5	0	741

$Sxlp^{e}$ tTa, tRe $Ras64B^{V12}$ on the 3rd chromosome

Tetracycline Conc. µg/ml	Female	Male
0.1	0	682
1	0	804
5	0	648

$Sxlp^{e}$ tTa, tRe $Msl\text{-}2^{Nopu}$ on the X chromosome

Tetracycline Conc. µg/ml	Female	Male
0.1	0	732
1	0	771
5	0	816

$Sxlp^{e}$ tTa, tRe $Msl\text{-}1^{Mpu}$ on the X chromosome

Tetracycline Conc. µg/ml	Female	Male
0.1	0	749
1	0	737
5	0	718

$Sxlp^{e}$ tTa, tRe $Msl\text{-}2^{Nopu}$ on the 3rd chromosome

Tetracycline Conc. µg/ml	Female	Male
0.1	0	696
1	0	658
5	0	711

Hsp26 tTa, tRe $Msl\text{-}2^{Nopu}$ on the 2nd chromosome

Tetracycline Conc. µg/ml	Female	Male
0.1	0	733
1	0	776
5	0	728

Hsp26 tTa, tRe Msl-1^{Mpu} on the 2^{nd} chromosome

Tetracycline Conc. µg/ml	Female	Male
0.1	0	765
1	0	702
5	0	773

Yp3 tTa, tRe Msl-2^{Nopu} on the 2^{nd} chromosome

Tetracycline Conc. µg/ml	Female	Male
0.1	0	799
1	0	749
5	0	744

Yp3 tTa, tRe Msl-1^{Mpu} on the X chromosome

Tetracycline Conc. µg/ml	Female	Male
0.1	0	718
1	0	753
5	0	757

Yp3 tTa, tRe Ras64B^{V12} on the 2^{nd} chromosome

Conclusions

At low temperature there is a slight leakiness, but only at a level of <1% escapers. All versions are extremely effective at 29°C. This is important as many of the most important target species for control are tropical and are grown in culture at around 28°C, e.g. *Ceratitis capitata, Anopheles gambiae, Aedes aegypti.*

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Claims

1. A multicellular organism carrying a dominant sex-specific lethal genetic system which is conditional.

2. A multicellular organism according to claim 1 which does not have a dominant sex-specific lethal genetic system which is unconditional and is expressed in every individual.

3. A method of biological control for an organism, the organism having discrete sexual entities, the method comprising the steps of:

1 production of a stock of genetically engineered organism according to claim 1;

2 release of the genetically engineered organism into the environment either as:

a) a population containing both sexes at a certain stage of the life cycle of the organism, in the knowledge that females will die and only males will mature into adults, or

b) a single sex population, i.e. after a sex specific dominant lethal effect has been expressed prior to release,

wherein one sexual entity is eliminated by expression of the conditional dominant sex specific lethal genetic system and the conditional expression of the lethal gene is such that the lethal effect occurs in the natural environment of the organism to cause the biological control.

4. A method of biological control, comprising:

breeding a stock of organisms according to claim 1 under permissive conditions, allowing the survival of organisms, to give a dual sex biological control agent;

optionally before the next step imposing or permitting restrictive conditions to cause death of entities of one sex and thereby providing a single sex biological control agent comprising entities of the other sex carrying the conditional dominant lethal genetic system;

releasing the dual sex or single sex biological control agent into the environment at a locus for biological control, and

achieving biological control through expression of the genetic system in offspring resulting from interbreeding of the individuals of the biological control agent with individuals of the opposite sex of the wild population.

5. A method according to claim 4, wherein the expression of the lethal genetic system occurs in the absence of a substance which is absent from the natural environment of the organism.

6. A biological control agent comprising a stock of organisms according to claim 1 having male and female entities.

7. A biological control agent comprising a stock of organisms according to claim 1 having male or female entities.

8. A biological control agent according to claim 6 or 7, wherein the organism is an insect.

9. A biological control agent according to claim 6 or 7, wherein the organism is an plant.

Amendments to the claims have been filed as follows

1. A non-human multicellular organism carrying a recombinant dominant lethal genetic system, the lethal effect of which is conditional, wherein the lethal effect of the lethal system occurs in the natural environment of the organism.

2. An organism according to claim 1, wherein the conditional dominant lethal genetic system is the only recombinant element present in the organism.

3. An organism according to claim 1 or 2, wherein the expression of the lethal genetic system occurs in the absence of a substance which is absent from the natural environment of the organism.

4. An organism according to claim 3, wherein the substance is a dietary additive which is not a normal food component for the organism.

5. An organism according to claim 4 wherein the dietary additive is tetracycline or a tetracycline analogue.

6. An organism according to any preceding claim wherein the lethal effect of the dominant lethal system is conditionally suppressible.

7. An organism according to any preceding claim, wherein the lethal system is homozygous at more than one locus.

8. An organism according to any preceding claim, wherein the lethal system is located on the X chromosome.

9. An organism according to any preceding claim, wherein the organism is an insect.

10. An organism according to claim 9, wherein the organism is selected from the group of: Japanese beetle (*Popilla japonica*),White-fringed beetle (*Graphognatus spp.*), Citrus blackfly (*Aleurocanthus woglumi*), Oriental fruit fly (*Dacus dorsalis*), Olive fruit fly *(Dacus oleae*), tropical fruit fly (*Dacus cucurbitae, Dacus zonatus*), Mediterranean fruit fly (*Ceratitis*

capitata), Natal fruit fly (*Ceratitis rosa*), Cherry fruit fly *(Rhagoletis cerasi*), Queensland fruit fly (*Bactrocera tryoni*), Caribbean fruit fly (*Anastrepha suspensa*), imported fire ants (*Solenopis richteri, Solenopis invicta*), Gypsy moth (*Lymantria dispar*), Codling moth (*Cydia pomonella*), Brown tail moth (*Euproctis chrysorrhoea*), yellow fever mosquito (*Aedes aegypti*), malaria mosquitoes (*Anopheles gambiae, Anopheles stephansi*), New world screwworm (*Cochliomyia hominivorax*), Old World Screwworm (*Chrysomya bezziana*), Tsetse fly (*Glossina spp*), Boll weevil (*Anthonomous grandis*), Damsel fly (*Enallagma hageni*), Dragonfly (*Libellula luctuosa*) and rice stem borer (*Tryporyza incertulas*).

11 An organism according to any of claims 1-8, wherein the organism is a plant.

12. An organism according to any preceding claim, wherein the lethal genetic system is sex-specific.

13. An organism according to claim 12, wherein the lethal effect is sex-specific at one life cycle stage, but not at another.

14 An organism according to claims 11or 12 wherein the organism does not have a dominant sex-specific lethal genetic system which is unconditional and is expressed in every individual.

15 An organism according to any of claims 11- 14, wherein the lethal effect is specifc to females or female tissue.

16 A biological control agent comprising a stock of organisms according to claim 12 having male and female entities.

17 A biological control agent comprising a stock of organisms according to claim 12 having male or female entities.

18 A method of biological control, comprising:

i breeding a stock of males and female organisms under permissive conditions, allowing the survival of males and females, to give a dual sex biological control agent;

ii releasing the dual sex or single sex biological control agent into the environment at a locus for biological control, and

iii achieving biological control through expression of the genetic system in offspring resulting from interbreeding of the individuals of the biological control agent with individuals of the opposite sex of the wild population.:

19 A method of biological control according to claim 18, wherein both males and females are distributed.

20 A method of biological control according to claim 18, wherein one sex is distributed.

21 A method of biological control according to claim 20, wherein there is sex-separation prior to organism distribution by expression of a sex specific lethal genetic system of an organism according to claims 12, 13 or 15.

22 A method of biological control according to any of claims 18-21, wherein the lethal effect results in killing of greater than 90% of the target class of the progeny of matings between released organisms and the wild population.

23 A method for sex selection, comprising expression of the lethal effect of a sex specific conditional lethal system according to claim 12 to eliminate one sex to leave an individual male or female population.

24 A vector suitable for use in the production of a recombinant multicellular organism as described in claims 1-15.

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Other: Online: AGRICOLA; BIOSIS; BIOTECHABS; CAPLUS; CONFSCI; CROPB; EPODOC; JAPIO; NTIS; LIFESCI; WPI

Documents considered to be relevant:

Category	Identity of document and relevant passage	Relevant to claims
X	US 5254801 (Monsanto Company) (See column 7, lines 21-24; 41-51; column 9, lines 39-54; Example 2)	1, 2
A	Science, Vol. 287, 31 March 2000, Thomas et al., pages 2474-2476 (See whole document)	1-9

X	Document indicating lack of novelty or inventive step	A	Document indicating technological background and/or state of the art.
Y	Document indicating lack of inventive step if combined with one or more other documents of same category.	P	Document published on or after the declared priority date but before the filing date of this invention.
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